

Northumbria Research Link

Citation: Voulgaridou, Georgia-Persephoni, Kiziridou, Magdalini, Mantso, Theodora, Chlichlia, Katerina, Galanis, Alex, Koukourakis, Michael, Franco, Rodrigo, Panagiotidis, Mihalís and Pappa, Aglaia (2016) Aldehyde dehydrogenase 3A1 promotes multi-modality resistance and alters gene expression profile in human breast adenocarcinoma MCF-7 cells. The International Journal of Biochemistry & Cell Biology, 77 (A). pp. 120-128. ISSN 1357-2725

Published by: Elsevier

URL: <http://dx.doi.org/10.1016/j.biocel.2016.06.004>
<<http://dx.doi.org/10.1016/j.biocel.2016.06.004>>

This version was downloaded from Northumbria Research Link:
<http://nrl.northumbria.ac.uk/id/eprint/29181/>

Northumbria University has developed Northumbria Research Link (NRL) to enable users to access the University's research output. Copyright © and moral rights for items on NRL are retained by the individual author(s) and/or other copyright owners. Single copies of full items can be reproduced, displayed or performed, and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided the authors, title and full bibliographic details are given, as well as a hyperlink and/or URL to the original metadata page. The content must not be changed in any way. Full items must not be sold commercially in any format or medium without formal permission of the copyright holder. The full policy is available online: <http://nrl.northumbria.ac.uk/policies.html>

This document may differ from the final, published version of the research and has been made available online in accordance with publisher policies. To read and/or cite from the published version of the research, please visit the publisher's website (a subscription may be required.)



**Northumbria
University**
NEWCASTLE



UniversityLibrary

**Aldehyde dehydrogenase 3A1 promotes multi-modality resistance and alters
gene expression profile in human breast adenocarcinoma MCF-7 cells**

Georgia-Persephoni Voulgaridou¹, Magdalini Kiziridou¹, Theodora Mantso^{1,4},
Katerina Chlichlia¹, Alex Galanis¹, Michael I. Koukourakis², Rodrigo Franco³,
Mihalis I. Panayiotidis⁴ and Aglaia Pappa^{1*}

Departments of ¹Molecular Biology & Genetics, and ²Radiotherapy/Oncology,
Radiobiology & Radiopathology Unit, Department of Medicine, School of Health
Sciences, Democritus University of Thrace, Alexandroupolis 68100, Greece; ³Redox
Biology Center, School of Veterinary Medicine & Biomedical Sciences, University of
Nebraska-Lincoln, Lincoln, NE 68583, USA, ⁴School of Life Sciences, Heriot-Watt
University, Edinburgh, EH14 4AS, Scotland, UK

*Corresponding author:

Aglaia Pappa, Ph.D.
Department of Molecular Biology & Genetics
Democritus University of Thrace
University Campus, Dragana,
68100 Alexandroupolis, GREECE
Phone: +30-25510-30625
Fax: +30-25510-30625
E-mail: apappa@mbg.duth.gr

Abstract

Aldehyde dehydrogenases participate in a variety of cellular homeostatic mechanisms like metabolism, proliferation, differentiation, apoptosis, whereas recently, they have been implicated in normal and cancer cell stemness. We explored roles for ALDH3A1 in conferring resistance to chemotherapeutics/radiation/oxidative stress and whether ectopic overexpression of ALDH3A1 could lead to alterations of gene expression profile associated with cancer stem cell-like phenotype. MCF-7 cells were stably transfected either with an empty vector (mock) or human aldehyde dehydrogenase 3A1 cDNA. The expression of aldehyde dehydrogenase 3A1 in MCF-7 cells was associated with altered cell proliferation rate and enhanced cell resistance against various chemotherapeutic drugs (4-hydroxyperoxycyclophosphamide, doxorubicin, etoposide, and 5-fluorouracil). Aldehyde dehydrogenase 3A1 expression also led to increased tolerance of MCF-7 cells to gamma radiation and hydrogen peroxide-induced stress. Furthermore, aldehyde dehydrogenase 3A1-expressing MCF-7 cells exhibited gene up-regulation of cyclins A, B1, B2, and down-regulation of cyclin D1 as well as transcription factors p21, CXR4, Notch1, SOX2, SOX4, OCT4, and JAG1. When compared to mock cells, no changes were observed in mRNA levels of ABCA2 and ABCB1 protein pumps with only a minor decrease of the ABCG2 pump in the aldehyde dehydrogenase 3A1-expressing cells. Also, the adhesion molecules EpCAM and CD49F were also found to be up-regulated in aldehyde dehydrogenase 3A1-expressing cells. Taken together, ALDH3A1 confers a multi-modality resistance phenotype in MCF-7 cells associated with slower growth rate, increased clonogenic capacity, and altered gene expression profile, underlining its significance in cell homeostasis.

51 **Keywords**

52 ALDH, ALDH3A1, MCF-7, cancer stem cells, oxidative stress, CD49F, EpCAM,

53 breast cancer, chemoresistance.

54

1. Introduction

Aldehyde dehydrogenase 3A1 (ALDH3A1) belongs to the broad family of aldehyde dehydrogenases (ALDHs). It is an NADP⁽⁺⁾-dependent enzyme, responsible for oxidizing medium chain saturated and unsaturated aldehydes to their corresponding carboxylic acids (Kim et al., 2014, Vasiliou et al., 2004, Vasiliou et al., 2000). Because of its ability to detoxify toxic aldehydes, by-products of lipid peroxidation like 4-hydroxy-2-nonenal (4-HNE), ALDH3A1 is considered an important component of cellular anti-oxidant defense (Black et al., 2012, Jang et al., 2014, Pappa et al., 2003a, Pappa et al., 2003b, Voulgaridou et al., 2011). Apart from its essential metabolic function, it has been suggested that ALDH3A1 may have additional roles in cellular homeostasis (Kim, Lee, 2014, Voulgaridou et al., 2013) including those of cell cycle regulation and protection against apoptosis and DNA damage (Chen et al., 2013, Estey et al., 2007, Jang, Bruse, 2014, Lassen et al., 2007, Pappa et al., 2005, Pappa et al., 2001, Stagos et al., 2010). However, ALDHs have gained even more attention, after their correlation with normal and cancer stem cell (CSC) populations (Gasparetto et al., 2012). In particular, the aldehyde dehydrogenase 1 (ALDH1) isoform was found to be critical for the isolation of cancer cells with stem-like features like self-renewal capacity, low proliferation rate, chemo-/radioresistance and enhanced clonogenic and tumorigenic potential (Calderaro et al., 2014, Croker and Allan, 2012, Deng et al., 2010, Lee et al., 2011, Sullivan et al., 2010, Yan et al., 2014). Moreover, increased expression of ALDH was also used as an index for the isolation of tumor cell subpopulations with stem-like characteristics in addition to being associated with poor clinical outcome (Lee, Kim, 2011, Sullivan, Spinola, 2010). In this context, ALDH3A1 has been described as “tumor-associated aldehyde dehydrogenase” (T-ALDH) (Lin et al., 1988) and has been shown to be upregulated in

several cancer types (Parajuli et al., 2014, Patel et al., 2008). Finally, only recently, it has been postulated to possess additional functional roles in stem cell biology in respect to self-protection, differentiation and cellular expansion (Ma and Allan, 2011). However, there are not many studies suggesting how exactly the over-expression of ALDH is utilized as a CSC marker and in particular what might be the underlying mechanism(s) of such involvement. For these reasons, we established an isogenic MCF-7 cell line pair (differing only in the expression of human ALDH3A1) with the aim to (i) investigate into the effects of ALDH3A1 on cell viability and colony formation efficiency under various exogenous stresses, like chemotherapeutics, hydrogen peroxide (H₂O₂) and gamma-irradiation) and (ii) to identify specific gene profiles attributed to such acquired CSC-like traits.

2. Materials and Methods

2.1 Materials

Human breast adenocarcinoma cell line MCF-7 was purchased from ATCC (Manassas, VA, USA). All of the standard culture media, fetal bovine serum (FBS), antibiotics and trypsin were either from Gibco (Life Technologies, Carlsbad, CA, USA), Biosera (East Sussex, UK), Biochrome (Berlin, Germany) or Sigma-Aldrich Co. (Taufkirchen, Germany). Lipofectamine and related transfection reagents were obtained from Life Technologies (Carlsbad, CA, USA) while hygromycin and protease inhibitors were from Carl Roth GmbH (Karlsruhe, Germany). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA) and chemiluminescence reagents and BCA Protein assay kit were from Thermo Scientific (Rockford, IL, USA). Autoradiography films were obtained from Genesee Scientific (San Diego, CA, USA). All chemotherapeutic agents were from Sigma-

Aldrich Co. (Taufkirchen, Germany) except 4-hydroxyperoxycyclophosphamide which was obtained from SantaCruz (Santa Cruz, California). Primers, dNTPs, Trizol and Platinum SYBR Green, were purchased from Invitrogen (Life Technologies Carlsbad, CA, USA) while random hexamers and PrimeScript Reverse Transcriptase were from Takara (Shiga, Japan). Rabbit polyclonal antibody against human ALDH3A1 was obtained from Abgent (San Diego, CA, USA). Mouse monoclonal antibody against EpCAM was from Cell Signalling Technology (Danvers, MA, USA). Goat anti-rabbit and mouse IgG horseradish peroxidase conjugated antibodies were obtained from Millipore (Bedford, MA, USA). CF488A goat anti-mouse IgG for immunofluorescence was from Biotium (Hayward, CA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany), Carl Roth GmbH (Karlsruhe, Germany) or Applichem (Darmstadt, Germany).

2.2 Cell Culture

Human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100µg/ml streptomycin, and 100units/ml penicillin. MCF-7 stable transfected cell lines were cultured in the same medium in the presence of 0.2mg/ml hygromycin. Cells were cultivated at 37°C with 5% CO₂ in a humidified incubator.

2.3 Stable Transfection

The full-length human ALDH3A1 was subcloned into a suitable mammalian expression vector constructed as previously described (Bunting and Townsend, 1996a,b, Pappa, Chen, 2003a). MCF-7 cells (10^6) were transfected with 16 μ g ALDH3A1/vector or control vector using the Lipofectamine 2000 reagent. Stably transfected cells were selected in the presence of 0.2mg/ml of hygromycin in the culture medium 48h post transfection. Selected clones were isolated, expanded and maintained in the presence of hygromycin.

2.4 Immunoblot analysis

Cell lysates were prepared in 50mM Tris-HCl, pH 8.0 containing NaCl, 1% Nonidet P₄₀, and the protease inhibitors: 100 μ g/ml PMSF, 0.5 μ g/ml leupeptin, 0.5 μ g/ml aprotinin and 1 μ g/ml pepstatin A. Protein concentration was determined by the BCA assay. Cell lysates (30 μ g of total protein) were separated by SDS-PAGE electrophoresis and transferred to 0.2 μ M PVDF membranes. The blots were blocked with 5% (w/v) BSA in TBST buffer (100mM Tris, pH 7.5, containing 150mM NaCl, and 0.1% v/v Tween-20) (blocking buffer) for 2 hours. Primary antibodies were used at different dilutions as follows: Polyclonal anti-ALDH3A1 and monoclonal anti-EpCAM were used at dilutions of 1:500 and 1:5000 in blocking buffer respectively (overnight, 4°C). Secondary horseradish peroxidase conjugated goat anti-rabbit and mouse antibodies were used at a dilution of 1:5000 in blocking buffer (1-hour incubation, RT). Signals were detected using the Supersignal West Pico Chemiluminescent Substrate.

2.5 Aldehyde dehydrogenase enzymatic activity assay

The enzymatic activity of ALDH3A1 was estimated as described previously (Pappa, Estey, 2003b). Briefly, a mixture of 75mM Na-pyrophosphate, pH 8.0 containing 1mM pyrazole, 2.5mM NADP⁺ and 50µl of cell lysates was prepared and used as a blank. The reaction was initiated by the addition of 0.5mM benzaldehyde. NADPH production was monitored for 5 min by the increase in the absorbance at 340nm with a Biochrom Libra S22 UV/visible spectrophotometer (Biochrom, Cambridge, UK). Finally, ALDH3A1 enzymatic activity was expressed as nanomoles of NADPH produced per minute, per mg of protein by taking into consideration the molar extinction coefficient of NADPH (6.22mM⁻¹/cm⁻¹).

2.6 Colony Formation Assay

Approximately 600 cells were plated in 10-cm culture dishes and subjected to various doses (0 to 10 Gray) of gamma radiation (Cobalt 60). Subsequently, cells were placed in a humidified incubator (37°C, 5% CO₂) and were monitored on a daily basis up to the formation of visible colonies (usually two weeks later). Cells were then fixed and stained with 0.5% of crystal violet solution diluted in 25% methanol. Colonies containing ≥50 of cells were counted using a stereomicroscope and digital images were obtained by camera or scanner and counted using ImageJ software.

2.7 Sulforhodamine B (SRB) assay

SRB assay was conducted as described earlier (Lassen et al. , 2006). Briefly, MCF-7/mock and MCF-7/ALDH3A1 cells were seeded in 96-well culture plates and then

were treated, in triplicates, with 4-hydroxyperoxycyclophosphamide, etoposide, doxorubicin, 5-fluorouracil, and H₂O₂. All chemotherapeutic agents were initially prepared in DMSO (or water in the case 4-hydroxyperoxycyclophosphamide) (as stock solutions of 50mM) and subsequently diluted (in cell culture medium) into various working concentrations: 4-hydroxyperoxycyclophosphamide (0-1600μM), etoposide (0-500μM), doxorubicin (0-1000μM), 5-fluorouracil (0-175μM). The working concentrations of H₂O₂ were 0-1000μM, and water was used as a vehicle. After a 72-h incubation, cells were fixed with 50% (w/v) trichloroacetic acid (TCA) for 1h at 4°C, washed 5 times with water and stained with 0.4% (w/v) SRB diluted in 1% acetic acid for 30 min. The excess dye was removed by washing with 1% (v/v) acetic acid. Plates were dried overnight, and the protein-bound dye was dissolved in 10mM Tris base solution. Optical density was determined at 492nm by using a microplate reader (Tecan Xflour 4). Controls were vehicle-treated cells. Sigma Plot software (version 10) was used for estimating the EC₅₀ values through the regression analysis *via* the four-parameter logistic curve as previously described (Anestopoulos et al., 2013).

2.8 Real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. For cDNA synthesis, 4.5μg of total RNA with 1 mM dNTPs and 50pmol of random hexamers were used. For real-time PCR analysis, Platinum SYBR Green was used according to the manufacturer's instructions. Reactions were carried out on an Applied Biosystems Step One Instrument. The sequences of the primers are provided in Table 1. Reactions were run in triplicate in three independent

experiments. Expression data were normalized to beta-actin using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen, 2001.

2.9 Immunofluorescence

Cells (1.5×10^5) grown in a monolayer on the surface of coverslips were fixed 24-h post plating with 4% formaldehyde in phosphate-buffered saline (PBS) (for 20 min) and washed three times with PBS. Formaldehyde was neutralized by the addition of 1M of Glycine (pH 8.5). Cells were permeabilized with 0.1% Triton X-100 followed by blocking with 5% BSA in PBS. The primary anti-EpCAM antibody was used at a dilution of 1:800 (1h, RT) whereas the secondary (CF488A goat anti-mouse) was used at a dilution of 1:250, in PBS, for 30 min. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (1 μ g/ml) and washed three times with PBS. Finally, cells were mounted with MOWIOL (Calbiochem, Bad Soden, Germany) and imaged with a 60x/NA 1.45 oil immersion objective and an Andor Ixon+885 digital camera on a customized Andor Revolution Spinning Disk Confocal System built around an IX81; Olympus stand (CIBIT Facility, MBG-DUTH). Andor IQ 2.7.1 software was used for image acquisition and analysis.

2.10 Statistical analysis

At least three independent experiments were conducted per sample for each condition tested. All values were expressed as mean \pm S.E. Comparison of results between two groups was performed by Student's *t*-test. Differences between

individual groups were assessed by a Dunnett post hoc test. Prism software (version 5) was used for all statistical analyses. A value of $p < 0.05$ was considered significant.

3. Results

3.1 Generation and characterization of the MCF-7 isogenic cell line pair

Stable transfection of the human ALDH3A1 cDNA in MCF-7 cells resulted in the selection of two ALDH3A1/MCF-7 clones (Figure 1). Clone #2 with the highest ALDH3A1 expression levels (confirmed by western blot analysis; Figure 1A) was chosen for all subsequent experiments and thus designated as ALDH3A1/MCF-7. Furthermore, expression of ALDH3A1 was also confirmed by real-time PCR (>100 -fold in mRNA levels compared to mock/ALDH3A1 cells; Figure 1B). Enzymatic activity, in ALDH3A1/MCF-7 cells, was estimated to be 535 ± 16 units/min/mg whereas Mock/ALDH3A1 cells exhibited negligible activity (Figure 1C). Regular monitoring of the enzymatic activity confirmed the maintenance of stable ALDH3A1 expression. Finally, it was observed that ALDH3A1/MCF-7 cells had considerably slower cycling capacity when compared to mock ones and estimated that their colony formation efficiency was approximately 57% of that of control cells (Figure 1D).

3.1 Expression of ALDH3A1 confers chemoresistance to MCF-7 cells

Next, we sought to determine the response of this isogenic cell line pair to various chemotherapeutic agents characterized by different modes of actions. Mock/ and ALDH3A1/MCF-7 cells were incubated for 72 h with increasing concentrations of 4-hydroxyperoxycyclophosphamide (an active derivative of cyclophosphamide), doxorubicin, etoposide, 5-fluorouracil and SRB-based cell viability curves were

plotted (Figures 2A-D respectively). Our data demonstrate that ALDH3A1 was associated with a chemoresistant phenotype as indicated by the cell viability curves in ALDH3A1-expressing cells compared to the non-expressing (mock) cells, under all treatments. ALDH3A1/MCF-7 cells exhibited approximately 2-fold resistance to 4-hydroxyperoxycyclophosphamide, (Figure 1A), ~11-fold resistance to doxorubicin (Figure 2B), 8-fold resistance to etoposide (Figure 2C), and 2-fold resistance to 5-fluorouracil (Figure 2D) when compared to mock cells.

3.2 Expression of ALDH3A1 confers resistance to radiation- and H₂O₂-induced cytotoxicity

Next, we investigated on the response of the isogenic cell line pair to other cytotoxic agents like H₂O₂ and gamma radiation. ALDH3A1 expression was associated with increased tolerance to H₂O₂-induced cytotoxicity (Figure 3A). Interestingly, following 72 h incubation with a range of H₂O₂ concentrations (up to 1mM) viability in ALDH3A1/MCF-7 cells did not fall below 60% when compared to control (untreated) cells. On the contrary, mock/MCF-7 cells sustained roughly 10% viability under the same experimental conditions (Figure 3A). Although the average EC₅₀ value for mock cells was estimated around 92μM, we were unable to calculate an accurate EC₅₀ value for ALDH3A1/MCF-7 cells in the same range of H₂O₂ concentrations (Figure 3A). Data from colony formation collected up to two weeks post-irradiation with a range of gamma irradiation (e.g. up to 10 Gy) revealed that ALDH3A1 contributed significantly to the maintenance of colony formation under radiation stress (Figure 3B).

3.3 ALDH3A1 alters gene expression profile in MCF-7 cells

The resistant phenotype of ALDH3A1/MCF-7 cells together with the observation of being slow cycling cells led to the evaluation of whether ALDH3A1 expression caused any alterations in the genetic make-up of MCF-7 cells. Thus, we analyzed the expression profile of several cell cycle regulatory proteins together with proteins-pumps that modulate drug import/export processes in the cell. Because slow cycling and chemotherapy/radiation resistance have been described as common traits for cancer stem cells (Alison et al., 2011, Ghaffari, 2011), we investigated the gene expression levels of those potentially relevant cancer stem cell markers including CXCR4, Notch1, SOX2, Oct4, JAG1, EpCAM, and CD49f. qRT-PCR experiments showed that the gene expression levels of cell cycle regulatory proteins (e.g. cyclins A, B1, and B2) were up-regulated while cyclin D and p21 were down-regulated. No significant changes were observed for cyclin E and p53 (Figure 4A). We also examined the effects of ALDH3A1 on the expression of the ATP-binding cassette (ABC) transporters ABCA2, ABCB1 (P-glycoprotein 1 or Multidrug Resistant Protein 1) and ABCG2 (Breast Cancer Resistance Protein 1). ALDH3A1 expression did not affect the expression levels of ABCA2 and ABCB1, whereas a slight decrease was observed for ABCG2 (Figure 4B). Significant changes were observed for all cancer stem cell markers tested in a manner where CXCR4, Notch1, SOX2, Oct4, and JAG1 were significantly down-regulated whereas the epithelial cell adhesion molecules EpCAM and CD49f (integrin subunit alpha 6) were up-regulated in ALDH3A1/MCF-7 cells (Figure 4C). To further validate the RT-PCR results, we selected the epithelial adhesion molecule EpCAM to confirm its up-regulation by both immunofluorescence and immunoblotting. Indeed, Figure 4D depicts enhanced

immunofluorescent localization of EpCAM in the ALDH3A1/MCF-7 cells while Western blotting also confirmed previous findings (Figure 4E).

4. Discussion

ALDHs represent a family of proteins implicated in cellular homeostasis in addition to their metabolic role (Pappa, Estey, 2003b). Indeed, a variety of ALDH isoforms are referred to as (i) corneal/lens crystallins (structural and protective components of cornea/lens) (Estey, Piatigorsky, 2007), (ii) cell protectors against ischemia-induced cardiac damage (Budass et al., 2010, Luo et al., 2014), (iii) modulators of cell proliferation rates (Lassen, Pappa, 2006, Liu et al., 2014, Pappa, Brown, 2005, Pappa, Chen, 2003a, Zhang et al., 2014) and (iv) mediators of differentiation in normal and cancer cells asserting to be markers of cell “stemness” (Balber, 2011, Dolle et al., 2015). In particular, correlation of ALDHs with normal/cancer stem cells is not recent with reports dating back to 1980s describing an association between leukemic cells overexpressing ALDHs and resistance to cyclophosphamide (Russo and Hilton, 1988, Tsukamoto et al., 1998). At the time and while studies were focused on the enzymatic activity specificities of ALDHs (capable of detoxifying cyclophosphamide), it was soon discovered that ALDHs expression was also a characteristic of healthy progenitor hematopoietic cells but was gradually lost during the maturation process to lymphocytes (Kastan et al., 1990). Since then, ALDHs (alone or in combination with other known markers) were considered a valuable marker for isolating hematopoietic progenitor populations (Armstrong et al., 2004, Fallon et al., 2003, Hess et al., 2004, Storms et al., 1999). Furthermore, their usage as a putative stem cell marker was also extended to the neuronal system (Balber, 2011, Cai et al., 2004, Corti et al., 2006a, Corti et al., 2006b). Less than a decade ago, ALDHs were studied more extensively

316 and thus were proposed as CSC markers, initially in leukemias and later in cases of
 317 solid tumours (Cheung et al., 2007, Pearce et al., 2005). Until now, ALDHs utilization
 318 as CSC markers have been investigated in a broad range of different cancers and in
 319 most cases, ALDHs expression was found to be a promising marker for the
 320 discrimination of sub-populations with stem-like characteristics (Chen et al., 2010,
 321 Deng, Yang, 2010, Emmink et al., 2011, Gong et al., 2010, Liang and Shi, 2012,
 322 Marcato et al., 2011, Shien et al., 2012, Sullivan, Spinola, 2010, Wang et al., 2012).
 323 On the other hand, there is still a long way to identifying specific ALDHs isoforms
 324 responsible for different types of cancer in addition to determining variable potential
 325 cancerous stem cell sub-population properties and qualities. Thus, elucidating the
 326 underlying mechanisms of ALDHs over-expression in CSCs is of crucial importance
 327 in tumor biology.

328 On another note, ALDH3A1 exhibits a distinct expression pattern. It is inducible by
 329 xenobiotics in the liver and constitutively expressed in certain epithelial tissues like
 330 lung, stomach, skin, and cornea. In the latter, its constitutive expression can reach up
 331 to 40% of the water-soluble proteins thus classifying ALDH3A1 as a corneal
 332 crystallin (Estey et al., 2010, Lassen, Bateman, 2007, Reisdorph and Lindahl, 2007).
 333 In fact, ALDH3A1 is a characteristic example of the multi-functional nature of the
 334 ALDH family as its expression has been associated with an apparent cell survival
 335 advantage under various stress conditions thus implicating ALDH3A1 as being a
 336 significant element in major homeostatic mechanisms including cell regulation and
 337 apoptosis (Estey, Piatigorsky, 2007, Pappa, Chen, 2003a). To examine the putative
 338 role of ALDH3A1 in the development of CSCs properties, we established MCF-7
 339 cells are over-expressing ALDH3A1 and studied its impact on stem cell-like
 340 properties. CSCs are relatively resistant to radiation as well as chemotherapeutic

agents like carboplatin, etoposide, fluorouracil, paclitaxel, daunorubicin, mitoxantrone, cyclophosphamide, temozolomide, and gemcitabine (Dylla et al., 2008, Hermann et al., 2007, Liu et al., 2006, Ma et al., 2008, Todaro et al., 2007, Wulf et al., 2001). Interestingly, our results indicated that ALDH3A1 protects MCF-7 cells from the cytotoxic effects of a wide variety of commonly used chemotherapeutic agents like 4-hydroxyperoxycyclophosphamide, etoposide, doxorubicin and 5-fluorouracil (Horak et al., 2013, Lekakis et al., 2012, Loi et al., 2013, Moitra et al., 2012). Indeed, previous studies have documented up-regulation of ALDHs with enhanced chemoresistance in breast cancer cells both *in vitro* and *in vivo* (Cioce et al., 2014, Croker and Allan, 2012, Lee, Kim, 2011). The results are in accordance with previous studies that have shown that overexpression of ALDH3A1 results in resistance to 4-hydroxyperoxycyclophosphamide and other active metabolites of cyclophosphamide [Bunting et al. J Biol Chem. 1994, 269: 23197-23203, Moreb et al., 2007). Interestingly, increased resistance to doxorubicin has also been associated with the ectopic expression of other ALDH members (Moreb et al., Chem. Biol. Interact., 2012, 195: 52-60), which is possibly mediated through indirect mechanisms by modulating oxidative stress response as previously reported for ALDH3A1 in relation to resistance to mitomycin C and etoposide (Pappa A et al., J. Biol. Chem. 2005, 280: 27998–28006). Moreover, ALDH3A1/MCF-7 cells exhibited enhanced survival and colony formation capacities in the presence of additional stress factors like gamma radiation and exposure to H₂O₂. Certainly, the specificity of ALDH3A1 for the metabolism and detoxification of cyclophosphamide (Bunting and Townsend, 1996b) and 4-HNE (Pappa, Estey, 2003b) is an important contributing factor underlining resistance, but its ability to protect adequately against a variety of other stressors supports the notion for an overall, multi-mode resistance phenotype

characteristic of ALDH3A1/MCF-7 cells. One possible mechanism accountable for the apparent resistance of these cells would be their slow-growing rate. This is in accordance with another study where ALDH3A1 led to inhibition of proliferation, slower cell cycling rates, and lower colony formation efficiency expression in human corneal epithelial cells (Estey, Piatigorsky, 2007, Pappa, Brown, 2005). This anti-proliferative action of ALDH3A1 was also observed in our study where the ALDH3A1/MCF-7 cells had the capacity to form only about 57% of the colonies formed in mock/MCF-7 cells. In general, CSCs are slow-growing cells in the quiescent state and consequently resistant to drugs designed to target fast-growing cancer cells (Dalerba et al., 2007, Tirino et al., 2013, Vinogradov and Wei, 2012). To characterize the molecular mechanisms responsible for the slow proliferation rates observed, we analyzed the gene expression profile of key cell cycle regulatory proteins. We noticed that ALDH3A1-expressing MCF-7 cells exhibited an (i) up-regulation of cyclins A, B1, B2 and (ii) down-regulation of cyclin D1 and transcription factor p21. Previous studies demonstrated that protein levels of cyclins A, B, E, E2F1, and p21, as well activities of cyclin A- and cyclin B- dependent kinases were all decreased in ALDH3A1/HCE cells (Pappa, Brown, 2005). While it is true that the comparative qPCR method used in this study detects differences only at the transcriptional level, the differential expression pattern of major cell cycle regulatory proteins (also previously reported for ALDH3A1-expressing HCE cells) may account for the slow proliferation phenotype observed. On the other hand, there are also reports associating knock down of ALDH3A1 in lung cancer cells with slower growth (Moreb et al., 2008). To this end, findings so far appear contradictory, and although they may reflect tissue-specific issues or differences in biology between

normal and cancer cells, they urge the need for further investigations towards the clarification of the role of ALDH3A1 in cell proliferation.

The possibility that drug resistance displayed by the ALDH3A1-expressing cells is likely due to enhanced expression of transporters that mediate chemotherapeutic drug efflux (Gottesman et al., 2002, Ween et al., 2015) was excluded. In general, several types of ABC transporters are known to be over-expressed in a variety of cancers where they are responsible for the development of chemoresistance (Chang et al., 2009, Doyle and Ross, 2003, Gottesman, Fojo, 2002, Mack et al., 2008). However, no detectable changes were observed in mRNA levels of ABCA2 and ABCB1 protein pumps. On the contrary, only a minor decrease observed for the ABCG2 pump in the ALDH3A1-expressing cells compared to mock. Another possible mechanism for the observed chemo-/radioresistance, in the presence of ALDH3A1, would be through mediating DNA damage checkpoint response. Indeed, increased activation of the DNA damage checkpoint response has been associated with expression of ALDH3A1 in corneal epithelial cells and preliminary data (obtained in our lab) certainly points towards this direction (*data not shown*). Similarly, the resistance of glioblastoma CSCs to irradiation has been attributed to increased activation of the DNA damage checkpoint (Bao et al., 2006).

To better characterize the changes caused by ALDH3A1 on gene expression, we investigated the presence of presumed protein markers found to be up-regulated in CSCs. The gene expression profile was significantly differentiated between the two MCF-7 isogenic cell lines. The mRNA levels of CXCR4, Notch1, SOX2, SOX4, OCT4, and JAG1, displayed down-regulation whereas EpCAM and CD49F were significantly up-regulated in the ALDH3A1/MCF-7 cells. We further validated the expression of the epithelial cell adhesion molecule (EpCAM) by immunofluorescence

and immunoblotting and showed that EpCAM protein levels were substantially elevated in the ALDH3A1 expressing MCF-7 cells. EpCAM together with CD49F have been studied extensively for their functional roles and usage as potential CSCs markers (Cariati et al., 2008, Deng et al., 2015, Guo et al., 2012, Guo et al., 2014, Wang et al., 2011). EpCAM is suggested to provide a sustained proliferative signal to cancer-initiating and normal stem cells where it is overexpressed. Cancer cells appear to benefit from the constitutive expression of EpCAM for proliferation, self-renewal, and anchorage-independent growth and invasiveness (Munz et al., 2009). On the other hand, CD49F (also known as $\alpha 6$ integrin) plays a significant role in cell adhesion. Its high expression in mammary epithelial cells is associated with progenitor and stem cell activity (Goel et al., 2014). This integrin acts as an adhesion receptor for the mammary epithelial cells mediating developmental signals and assisting cells in sensing growth factor and hormonal signals (Kaimala et al., 2012). It appears to play a major role in sustaining the survival of mammary carcinoma cells especially under stress conditions such as those existing in the tumor microenvironment (Chung and Mercurio, 2004).

In conclusion, MCF-7 cells over-expressing ALDH3A1 demonstrated low proliferation rates associated with a resistant phenotype against various sources of cell stress including exposure to various chemotherapeutics, gamma radiation, and H_2O_2 insult. Furthermore, they displayed differential expression of proteins involved in cell cycle regulation and increased expression of the cell adhesion molecules CD49f and EpCAM. Although the precise mechanisms remain unclear, our findings provide considerable implications on defining the biological significance of ALDH3A1 in cell homeostasis.

440 **Acknowledgments**

441 This research has been co-financed by the European Union (European Social Fund –
442 ESF) and Greek national funds through the Operational Program "Education and
443 Lifelong Learning" of the National Strategic Reference Framework (NSRF) -
444 Research Funding Program: Heracleitus II. Investing in knowledge society through
445 the European Social Fund.

446

447

448

449

FIGURE LEGENDS

Figure 1: Characterization of the MCF-7 isogenic cell line pair. **A.** Western blot analysis of ALDH3A1 expression: Lane 1: recombinant ALDH3A1 (1 μ g), lanes 2-7: 30 μ g cell extracts, 2; parental MCF-7, 3-4; mock-transfected ALDH3A1, 6-7: ALDH3A1/MCF-7 transfected clones. **B.** ALDH3A1 gene expression levels detected by real-time PCR in mock/ and ALDH3A1/MCF7. **C.** Enzymatic activity of ALDH3A1 in mock/MCF-7 and ALDH3A1/MCF-7 cells. Results are expressed as means of a minimum of three independent experiments \pm SE. **D.** Colony formation efficiency of mock and ALDH3A1/MCF-7 cells. Cells (600) were seeded in 10 cm culture dishes and were allowed to form colonies for two weeks in a humidified incubator that were subsequently counted following crystal violet staining by using Image J. Results are expressed as mean \pm S.E of three independent experiments. *** $p < 0.001$.

Figure 2: Effect of various chemotherapeutic agents on cell viability of mock/ and ALDH3A1/MCF-7 cells.

Viability curves of mock/ and ALDH3A1/MCF-7 cells along with the calculated half maximal effective concentrations (EC_{50} values) of (A) 4-hydroxyperoxycyclophosphamide, (B) doxorubicin, (C) etoposide, and (D) 5-fluorouracil are represented. Viability curves of the ALDH3A1/MCF-7 cells are shifted to the right indicating increased tolerance of the cells to the cytotoxic effect of the agents used. Results are shown as mean \pm S.E. At least three independent experiments were performed for each condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3: Effect of H₂O₂ and gamma radiation on the viability of mock/ and ALDH3A1/MCF-7 cells. The viability curves of mock/ and ALDH3A1/MCF7 cells along with the half maximal effective concentrations (EC₅₀ values) of (A) H₂O₂ and (B) gamma radiation are presented. ALDH3A1 expression is associated with increased tolerance to the cytotoxic effects of H₂O₂ and gamma radiation. Results are presented as mean ± SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 4: Expression of ALDH3A1 alters gene profiling in ALDH3A1/MCF-7 cells. Effect of ALDH3A1 on the gene expression of (A) cycle cell regulatory proteins (B) Membrane ABC transporters (C) Cancer stem cell markers. The comparative quantification $\Delta\Delta C_t$ method was utilized for analyzing the fold change of gene expression. Beta-actin gene was used as endogenous control for the normalization of samples. **D:** Immunofluorescence for EpCAM (green) in ALDH3A1/MCF-7 (i) and mock/MCF-7 (ii) cells. No secondary antibody for EpCAM was used in the negative control (iii), whereas nuclei were stained with DAPI (4',6-diamino-2-phenylindole) (blue). **E.** Western blotting analysis for EpCAM in mock and ALDH3A1/MCF-7 cells. Results are shown as mean ± S.E. At least three independent experiments were performed for each condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 1. Primers used for the real-time PCR comparative quantification

GENE	FORWARD PRIMER	REVERSE PRIMER
β -actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTC
ALDH3A1	CAGCGGCATGGGATCCTA	GCGGCGGTGAGAGAAAGTC
Cyclin A	ACGGGTTGCACCCCTTAAG	CCAAGGAGGAACGGTGACA
Cyclin B1	GGCCTCTACCTTTGCACTTCCT	GCTCGACATCAACCTCTCCAA
Cyclin B2	AAGCTTTTTCTGATGCCTTGCT	AGGGTTCTCCCAATCTTCGTTAT
Cyclin D	AGACCTTCGTTGCCTCTTGCTG	ATGGAGGGCGGATTGGAA
Cyclin E	GGCCTTGATCATTCTCGTCAT	CGCACCACTGATACCCTGAA
p53	TCTGTCCCTTCCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
p21	GGCGGGCTGCATCCA	AGTGGTGTCTCGGTGACAAAGTC
ABCA2	AGATGGACAAGATGATCGAG	GCTTGACTTCAGGATGAGG
ABCB1	GAGGAAGACATGACCAGGTA	CTGTTCGATTATAGCATGAA
ABCG2	ACCTGAAGGCATTTACTGAA	TCTTTCCTTGCAGCTAAGAC
CXCR4	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
Notch1	GCACCTCAGCCTGCACAGT	CTGTGTTGCTGGAGCATCTTCT

SOX2	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
SOX4	CTGCGCCTCAAGCACATG	TTCTTCCTGGGCCGGTACT
Oct4	CGACCATCTGCCGCTTG	GCCGCAGCTTACACATGTTCT
JAG1	TGAAGTAGAAGAGGACGACATGGA	CGGCTGCTTGGCAAACC
EpCAM	TTATGATCCTGACTGCGATGAGA	GGTGCCGTTGCACTGCTT
CD49F	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT

497

498

499

500

501

502

503

504

505

506

507

508

509

510

511

References

- Alison MR, Lim SM, Nicholson LJ. Cancer stem cells: problems for therapy? *J Pathol.* 2011;223:147-61.
- Anestopoulos I, Kavo A, Tentes I, Kortsaris A, Panayiotidis M, Lazou A, et al. Silibinin protects H9c2 cardiac cells from oxidative stress and inhibits phenylephrine-induced hypertrophy: potential mechanisms. *J Nutr Biochem.* 2013;24:586-94.
- Armstrong L, Stojkovic M, Dimmick I, Ahmad S, Stojkovic P, Hole N, et al. Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. *Stem Cells.* 2004;22:1142-51.
- Balber AE. Concise review: aldehyde dehydrogenase bright stem and progenitor cell populations from normal tissues: characteristics, activities, and emerging uses in regenerative medicine. *Stem Cells.* 2011;29:570-5.
- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006;444:756-60.
- Black W, Chen Y, Matsumoto A, Thompson DC, Lassen N, Pappa A, et al. Molecular mechanisms of ALDH3A1-mediated cellular protection against 4-hydroxy-2-nonenal. *Free Radic Biol Med.* 2012;52:1937-44.
- Budas GR, Disatnik MH, Chen CH, Mochly-Rosen D. Activation of aldehyde dehydrogenase 2 (ALDH2) confers cardioprotection in protein kinase C epsilon (PKC ϵ) knockout mice. *J Mol Cell Cardiol.* 2010;48:757-64.
- Bunting KD, Townsend AJ. De novo expression of transfected human class 1 aldehyde dehydrogenase (ALDH) causes resistance to oxazaphosphorine anti-cancer alkylating agents in hamster V79 cell lines. Elevated class 1 ALDH activity is closely correlated with reduction in DNA interstrand cross-linking and lethality. *J Biol Chem.* 1996a;271:11884-90.
- Bunting KD, Townsend AJ. Protection by transfected rat or human class 3 aldehyde dehydrogenase against the cytotoxic effects of oxazaphosphorine alkylating agents in hamster V79 cell lines. Demonstration of aldophosphamide metabolism by the human cytosolic class 3 isozyme. *J Biol Chem.* 1996b;271:11891-6.
- Cai J, Cheng A, Luo Y, Lu C, Mattson MP, Rao MS, et al. Membrane properties of rat embryonic multipotent neural stem cells. *J Neurochem.* 2004;88:212-26.
- Calderaro J, Nault JC, Bioulac-Sage P, Laurent A, Blanc JF, Decaens T, et al. ALDH3A1 is overexpressed in a subset of hepatocellular carcinoma characterised by activation of the Wnt/ss-catenin pathway. *Virchows Arch.* 2014;464:53-60.
- Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C, et al. Alpha-6 integrin is necessary for the tumorigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. *Int J Cancer.* 2008;122:298-304.
- Chang H, Rha SY, Jeung HC, Im CK, Ahn JB, Kwon WS, et al. Association of the ABCB1 gene polymorphisms 2677G>T/A and 3435C>T with clinical outcomes of paclitaxel monotherapy in metastatic breast cancer patients. *Ann Oncol.* 2009;20:272-7.
- Chen Y, Thompson DC, Koppaka V, Jester JV, Vasiliou V. Ocular aldehyde dehydrogenases: protection against ultraviolet damage and maintenance of transparency for vision. *Prog Retin Eye Res.* 2013;33:28-39.
- Chen YW, Chen KH, Huang PI, Chen YC, Chiou GY, Lo WL, et al. Cucurbitacin I suppressed stem-like property and enhanced radiation-induced apoptosis in head and neck squamous carcinoma--derived CD44(+)ALDH1(+) cells. *Mol Cancer Ther.* 2010;9:2879-92.
- Cheung AM, Wan TS, Leung JC, Chan LY, Huang H, Kwong YL, et al. Aldehyde dehydrogenase activity in leukemic blasts defines a subgroup of acute myeloid leukemia with adverse prognosis and superior NOD/SCID engrafting potential. *Leukemia.* 2007;21:1423-30.
- Chung J, Mercurio AM. Contributions of the alpha6 integrins to breast carcinoma survival and progression. *Mol Cells.* 2004;17:203-9.
- Cioce M, Valerio M, Casadei L, Pulito C, Sacconi A, Mori F, et al. Metformin-induced metabolic reprogramming of chemoresistant ALDHbright breast cancer cells. *Oncotarget.* 2014;5:4129-43.

566 Corti S, Locatelli F, Papadimitriou D, Donadoni C, Del Bo R, Crimi M, et al. Transplanted
567 ALDHhiSSClo neural stem cells generate motor neurons and delay disease progression of
568 nmd mice, an animal model of SMARD1. *Hum Mol Genet.* 2006a;15:167-87.

569 Corti S, Locatelli F, Papadimitriou D, Donadoni C, Salani S, Del Bo R, et al. Identification of
570 a primitive brain-derived neural stem cell population based on aldehyde dehydrogenase
571 activity. *Stem Cells.* 2006b;24:975-85.

572 Croker AK, Allan AL. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces
573 chemotherapy and radiation resistance of stem-like ALDHhiCD44(+) human breast cancer
574 cells. *Breast Cancer Res Treat.* 2012;133:75-87.

575 Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med.*
576 2007;58:267-84.

577 Deng S, Yang X, Lassus H, Liang S, Kaur S, Ye Q, et al. Distinct expression levels and
578 patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human
579 epithelial cancers. *PLoS One.* 2010;5:e10277.

580 Deng Z, Wu Y, Ma W, Zhang S, Zhang YQ. Adoptive T-cell therapy of prostate cancer
581 targeting the cancer stem cell antigen EpCAM. *BMC Immunol.* 2015;16:1.

582 Dolle L, Boulter L, Leclercq IA, van Grunsven LA. Next generation of ALDH substrates and
583 their potential to study maturational lineage biology in stem and progenitor cells. *Am J*
584 *Physiol Gastrointest Liver Physiol.* 2015;308:G573-8.

585 Doyle L, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein
586 BCRP (ABCG2). *Oncogene.* 2003;22:7340-58.

587 Dylla SJ, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, et al. Colorectal cancer stem cells
588 are enriched in xenogeneic tumors following chemotherapy. *PLoS One.* 2008;3:e2428.

589 Emmink BL, Van Houdt WJ, Vries RG, Hoogwater FJ, Govaert KM, Verheem A, et al.
590 Differentiated human colorectal cancer cells protect tumor-initiating cells from irinotecan.
591 *Gastroenterology.* 2011;141:269-78.

592 Estey T, Chen Y, Carpenter JF, Vasiliou V. Structural and functional modifications of corneal
593 crystallin ALDH3A1 by UVB light. *PLoS One.* 2010;5:e15218.

594 Estey T, Piatigorsky J, Lassen N, Vasiliou V. ALDH3A1: a corneal crystallin with diverse
595 functions. *Exp Eye Res.* 2007;84:3-12.

596 Fallon P, Gentry T, Balber AE, Boulware D, Janssen WE, Smilee R, et al. Mobilized
597 peripheral blood SSCloALDHbr cells have the phenotypic and functional properties of
598 primitive haematopoietic cells and their number correlates with engraftment following
599 autologous transplantation. *Br J Haematol.* 2003;122:99-108.

600 Gasparetto M, Sekulovic S, Bocker C, Tang P, Zakaryan A, Xiang P, et al. Aldehyde
601 dehydrogenases are regulators of hematopoietic stem cell numbers and B-cell development.
602 *Exp Hematol.* 2012;40:318-29 e2.

603 Ghaffari S. Cancer, stem cells and cancer stem cells: old ideas, new developments. *F1000*
604 *Med Rep.* 2011;3:23.

605 Goel HL, Gritsko T, Pursell B, Chang C, Shultz LD, Greiner DL, et al. Regulated splicing of
606 the alpha6 integrin cytoplasmic domain determines the fate of breast cancer stem cells. *Cell*
607 *Rep.* 2014;7:747-61.

608 Gong C, Yao H, Liu Q, Chen J, Shi J, Su F, et al. Markers of tumor-initiating cells predict
609 chemoresistance in breast cancer. *PLoS One.* 2010;5:e15630.

610 Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent
611 transporters. *Nat Rev Cancer.* 2002;2:48-58.

612 Guo C, Liu H, Zhang BH, Cadaneanu RM, Mayle AM, Garraway IP. Epcam, CD44, and
613 CD49f distinguish sphere-forming human prostate basal cells from a subpopulation with
614 predominant tubule initiation capability. *PLoS One.* 2012;7:e34219.

615 Guo Z, Li LQ, Jiang JH, Ou C, Zeng LX, Xiang BD. Cancer stem cell markers correlate with
616 early recurrence and survival in hepatocellular carcinoma. *World J Gastroenterol.*
617 2014;20:2098-106.

618 Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, et al. Distinct populations
619 of cancer stem cells determine tumor growth and metastatic activity in human pancreatic
620 cancer. *Cell Stem Cell.* 2007;1:313-23.

Hess DA, Meyerrose TE, Wirthlin L, Craft TP, Herrbrich PE, Creer MH, et al. Functional
 characterization of highly purified human hematopoietic repopulating cells isolated according
 to aldehyde dehydrogenase activity. *Blood*. 2004;104:1648-55.
 Horak CE, Pusztai L, Xing G, Trifan OC, Saura C, Tseng LM, et al. Biomarker analysis of
 neoadjuvant doxorubicin/cyclophosphamide followed by ixabepilone or Paclitaxel in early-
 stage breast cancer. *Clin Cancer Res*. 2013;19:1587-95.
 Jang JH, Bruse S, Liu Y, Duffy V, Zhang C, Oyamada N, et al. Aldehyde dehydrogenase 3A1
 protects airway epithelial cells from cigarette smoke-induced DNA damage and cytotoxicity.
Free Radic Biol Med. 2014;68:80-6.
 Kaimala S, Bisana S, Kumar S. Mammary gland stem cells: more puzzles than explanations. *J*
Biosci. 2012;37:349-58.
 Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of
 elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood*.
 1990;75:1947-50.
 Kim SW, Lee J, Lee B, Rhim T. Proteomic analysis in pterygium; upregulated protein
 expression of ALDH3A1, PDIA3, and PRDX2. *Mol Vis*. 2014;20:1192-202.
 Lassen N, Bateman JB, Estey T, Kuszak JR, Nees DW, Piatigorsky J, et al. Multiple and
 additive functions of ALDH3A1 and ALDH1A1: cataract phenotype and ocular oxidative
 damage in *Aldh3a1(-)/Aldh1a1(-)* knock-out mice. *J Biol Chem*. 2007;282:25668-76.
 Lassen N, Pappa A, Black WJ, Jester JV, Day BJ, Min E, et al. Antioxidant function of
 corneal ALDH3A1 in cultured stromal fibroblasts. *Free Radic Biol Med*. 2006;41:1459-69.
 Lee HE, Kim JH, Kim YJ, Choi SY, Kim SW, Kang E, et al. An increase in cancer stem cell
 population after primary systemic therapy is a poor prognostic factor in breast cancer. *Br J*
Cancer. 2011;104:1730-8.
 Lekakis L, Tryfonopoulos D, Pistamatzi N, Panopoulos C, Koumakis G, Demiri S, et al.
 Salvage chemotherapy with cisplatin and 5-fluorouracil in metastatic breast cancer. Particular
 activity against liver metastases. *Anticancer Res*. 2012;32:1833-7.
 Liang D, Shi Y. Aldehyde dehydrogenase-1 is a specific marker for stem cells in human lung
 adenocarcinoma. *Med Oncol*. 2012;29:633-9.
 Lin KH, Brennan MD, Lindahl R. Expression of tumor-associated aldehyde dehydrogenase
 gene in rat hepatoma cell lines. *Cancer Res*. 1988;48:7009-12.
 Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, et al. Analysis of gene expression
 and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer*. 2006;5:67.
 Liu Y, Jiang X, Zeng Y, Zhou H, Yang J, Cao R. Proliferating pancreatic beta-cells
 upregulate ALDH. *Histochem Cell Biol*. 2014;142:685-91.
 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-8.
 Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, et al. Prognostic and predictive
 value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial
 in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with
 doxorubicin-based chemotherapy: BIG 02-98. *J Clin Oncol*. 2013;31:860-7.
 Luo XJ, Liu B, Ma QL, Peng J. Mitochondrial aldehyde dehydrogenase, a potential drug
 target for protection of heart and brain from ischemia/reperfusion injury. *Curr Drug Targets*.
 2014;15:948-55.
 Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells.
Stem Cell Rev. 2011;7:292-306.
 Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. CD133+ HCC cancer stem cells confer
 chemoresistance by preferential expression of the Akt/PKB survival pathway. *Oncogene*.
 2008;27:1749-58.
 Mack JT, Brown CB, Tew KD. ABCA2 as a therapeutic target in cancer and nervous system
 disorders. *Expert Opin Ther Targets*. 2008;12:491-504.
 Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, et al. Aldehyde dehydrogenase
 activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression
 is predictive of metastasis. *Stem Cells*. 2011;29:32-45.

675 Moitra K, Im K, Limpert K, Borsa A, Sawitzke J, Robey R, et al. Differential gene and
676 microRNA expression between etoposide resistant and etoposide sensitive MCF7 breast
677 cancer cell lines. *PLoS One*. 2012;7:e45268.

678 Moreb JS, Baker HV, Chang LJ, Amaya M, Lopez MC, Ostmark B, Chou W. ALDH
679 isozymes downregulation affects cell growth, cell motility and gene expression in lung cancer
680 cells. *Mol Cancer*. 2008;7:87. doi: 10.1186/1476-4598-7-87.

681 Moreb JS, Mohuczy D, Ostmark B, Zucali JR. RNAi-mediated knockdown of aldehyde
682 dehydrogenase class-1A1 and class-3A1 is specific and reveals that each contributes equally
683 to the resistance against 4-hydroperoxycyclophosphamide. *Cancer Chemother Pharmacol*.
684 2007;59:127-36.

685 Moreb JS, Ukar D, Moreb JS, Ucar D, Han S, Amory JK, Goldstein AS, Ostmark B, Chang
686 LJ. The enzymatic activity of human aldehyde dehydrogenases 1A2 and 2 (ALDH1A2 and
687 ALDH2) is detected by Aldefluor, inhibited by diethylaminobenzaldehyde and has significant
688 effects on cell proliferation and drug resistance. *Chem Biol Interact*. 2012;195:52-60.

689 Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell
690 signaling. *Cancer Res*. 2009;69:5627-9.

691 Pappa A, Brown D, Koutalos Y, DeGregori J, White C, Vasiliou V. Human aldehyde
692 dehydrogenase 3A1 inhibits proliferation and promotes survival of human corneal epithelial
693 cells. *J Biol Chem*. 2005;280:27998-8006.

694 Pappa A, Chen C, Koutalos Y, Townsend AJ, Vasiliou V. Aldh3a1 protects human corneal
695 epithelial cells from ultraviolet- and 4-hydroxy-2-nonenal-induced oxidative damage. *Free
696 Radic Biol Med*. 2003a;34:1178-89.

697 Pappa A, Estey T, Manzer R, Brown D, Vasiliou V. Human aldehyde dehydrogenase 3A1
698 (ALDH3A1): biochemical characterization and immunohistochemical localization in the
699 cornea. *Biochem J*. 2003b;376:615-23.

700 Pappa A, Sophos NA, Vasiliou V. Corneal and stomach expression of aldehyde
701 dehydrogenases: from fish to mammals. *Chem Biol Interact*. 2001;130-132:181-91.

702 Parajuli B, Georgiadis TM, Fishel ML, Hurley TD. Development of selective inhibitors for
703 human aldehyde dehydrogenase 3A1 (ALDH3A1) for the enhancement of cyclophosphamide
704 cytotoxicity. *Chembiochem*. 2014;15:701-12.

705 Patel M, Lu L, Zander DS, Sreerama L, Coco D, Moreb JS. ALDH1A1 and ALDH3A1
706 expression in lung cancers: correlation with histologic type and potential precursors. *Lung
707 Cancer*. 2008;59:340-9.

708 Pearce DJ, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA, et al. Characterization
709 of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid
710 leukemia samples. *Stem Cells*. 2005;23:752-60.

711 Reisdorph R, Lindahl R. Constitutive and 3-methylcholanthrene-induced rat ALDH3A1
712 expression is mediated by multiple xenobiotic response elements. *Drug Metab Dispos*.
713 2007;35:386-93.

714 Russo JE, Hilton J. Characterization of cytosolic aldehyde dehydrogenase from
715 cyclophosphamide resistant L1210 cells. *Cancer Res*. 1988;48:2963-8.

716 Shien K, Toyooka S, Ichimura K, Soh J, Furukawa M, Maki Y, et al. Prognostic impact of
717 cancer stem cell-related markers in non-small cell lung cancer patients treated with induction
718 chemoradiotherapy. *Lung Cancer*. 2012;77:162-7.

719 Stagos D, Chen Y, Cantore M, Jester JV, Vasiliou V. Corneal aldehyde dehydrogenases:
720 multiple functions and novel nuclear localization. *Brain Res Bull*. 2010;81:211-8.

721 Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, et al. Isolation of
722 primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity.
723 *Proc Natl Acad Sci U S A*. 1999;96:9118-23.

724 Sullivan JP, Spinola M, Dodge M, Raso MG, Behrens C, Gao B, et al. Aldehyde
725 dehydrogenase activity selects for lung adenocarcinoma stem cells dependent on notch
726 signaling. *Cancer Res*. 2010;70:9937-48.

727 Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, et al. Cancer stem cells
728 in solid tumors: an overview and new approaches for their isolation and characterization.
729 *FASEB J*. 2013;27:13-24.

Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell*. 2007;1:389-402.

Tsukamoto N, Chen J, Yoshida A. Enhanced expressions of glucose-6-phosphate dehydrogenase and cytosolic aldehyde dehydrogenase and elevation of reduced glutathione level in cyclophosphamide-resistant human leukemia cells. *Blood Cells Mol Dis*. 1998;24:231-8.

Vasiliou V, Pappa A, Estey T. Role of human aldehyde dehydrogenases in endobiotic and xenobiotic metabolism. *Drug Metab Rev*. 2004;36:279-99.

Vasiliou V, Pappa A, Petersen DR. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. *Chem Biol Interact*. 2000;129:1-19.

Vinogradov S, Wei X. Cancer stem cells and drug resistance: the potential of nanomedicine. *Nanomedicine (Lond)*. 2012;7:597-615.

Voulgaridou GP, Anestopoulos I, Franco R, Panayiotidis MI, Pappa A. DNA damage induced by endogenous aldehydes: current state of knowledge. *Mutat Res*. 2011;711:13-27.

Voulgaridou GP, Mantso T, Chlichlia K, Panayiotidis MI, Pappa A. Efficient *E. coli* expression strategies for production of soluble human crystallin ALDH3A1. *PLoS One*. 2013;8:e56582.

Wang Y, Shenouda S, Baranwal S, Rathinam R, Jain P, Bao L, et al. Integrin subunits alpha5 and alpha6 regulate cell cycle by modulating the chk1 and Rb/E2F pathways to affect breast cancer metastasis. *Mol Cancer*. 2011;10:84.

Wang YC, Yo YT, Lee HY, Liao YP, Chao TK, Su PH, et al. ALDH1-bright epithelial ovarian cancer cells are associated with CD44 expression, drug resistance, and poor clinical outcome. *Am J Pathol*. 2012;180:1159-69.

Ween MP, Armstrong MA, Oehler MK, Ricciardelli C. The role of ABC transporters in ovarian cancer progression and chemoresistance. *Crit Rev Oncol Hematol*. 2015.

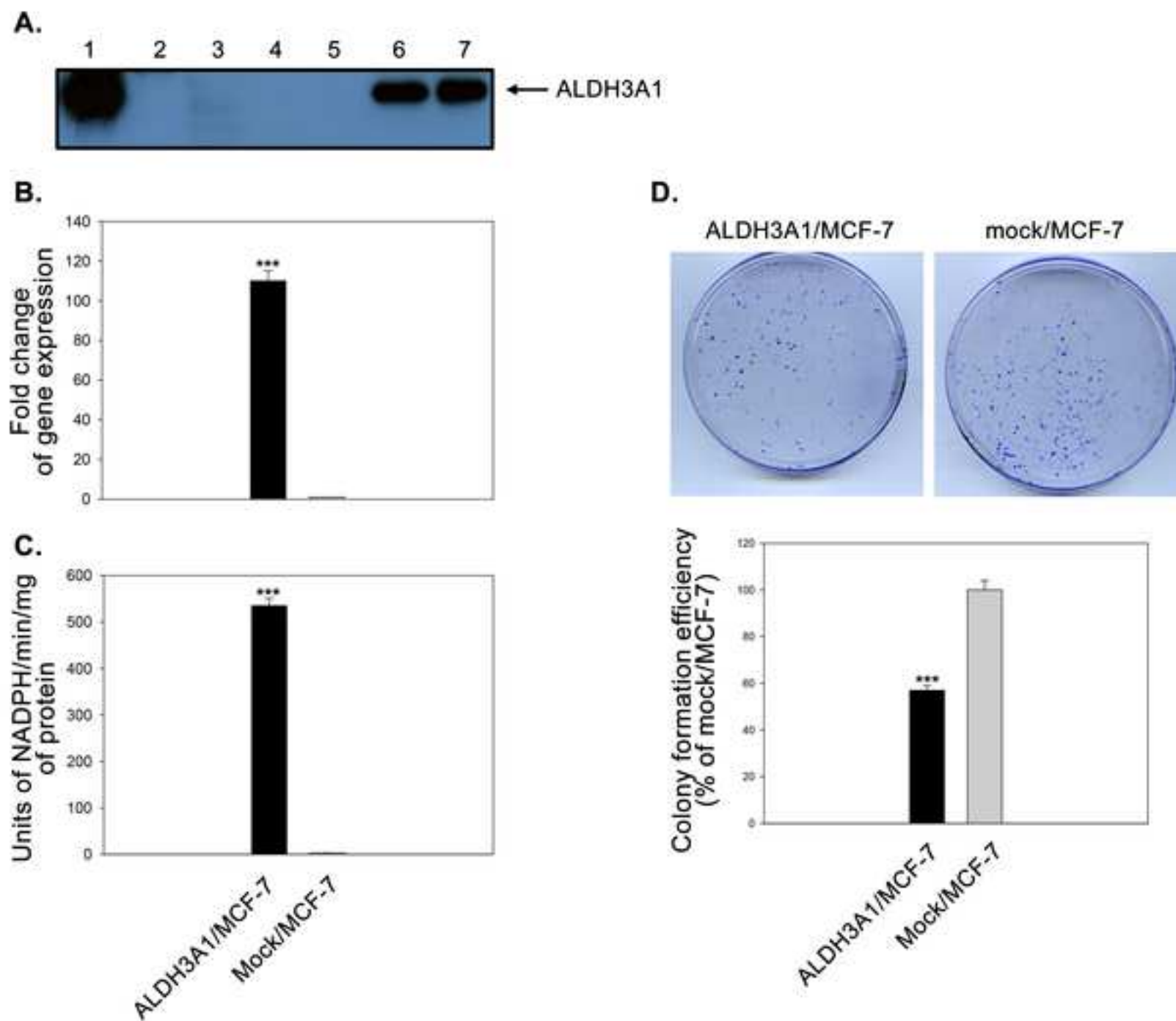
Wulf GG, Wang RY, Kuehnle I, Weidner D, Marini F, Brenner MK, et al. A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood*. 2001;98:1166-73.

Yan J, De Melo J, Cutz JC, Aziz T, Tang D. Aldehyde dehydrogenase 3A1 associates with prostate tumorigenesis. *Br J Cancer*. 2014;110:2593-603.

Zhang L, Wang L, Liu X, Zheng D, Liu S, Liu C. ALDH expression characterizes G1-phase proliferating beta cells during pregnancy. *PLoS One*. 2014;9:e96204.

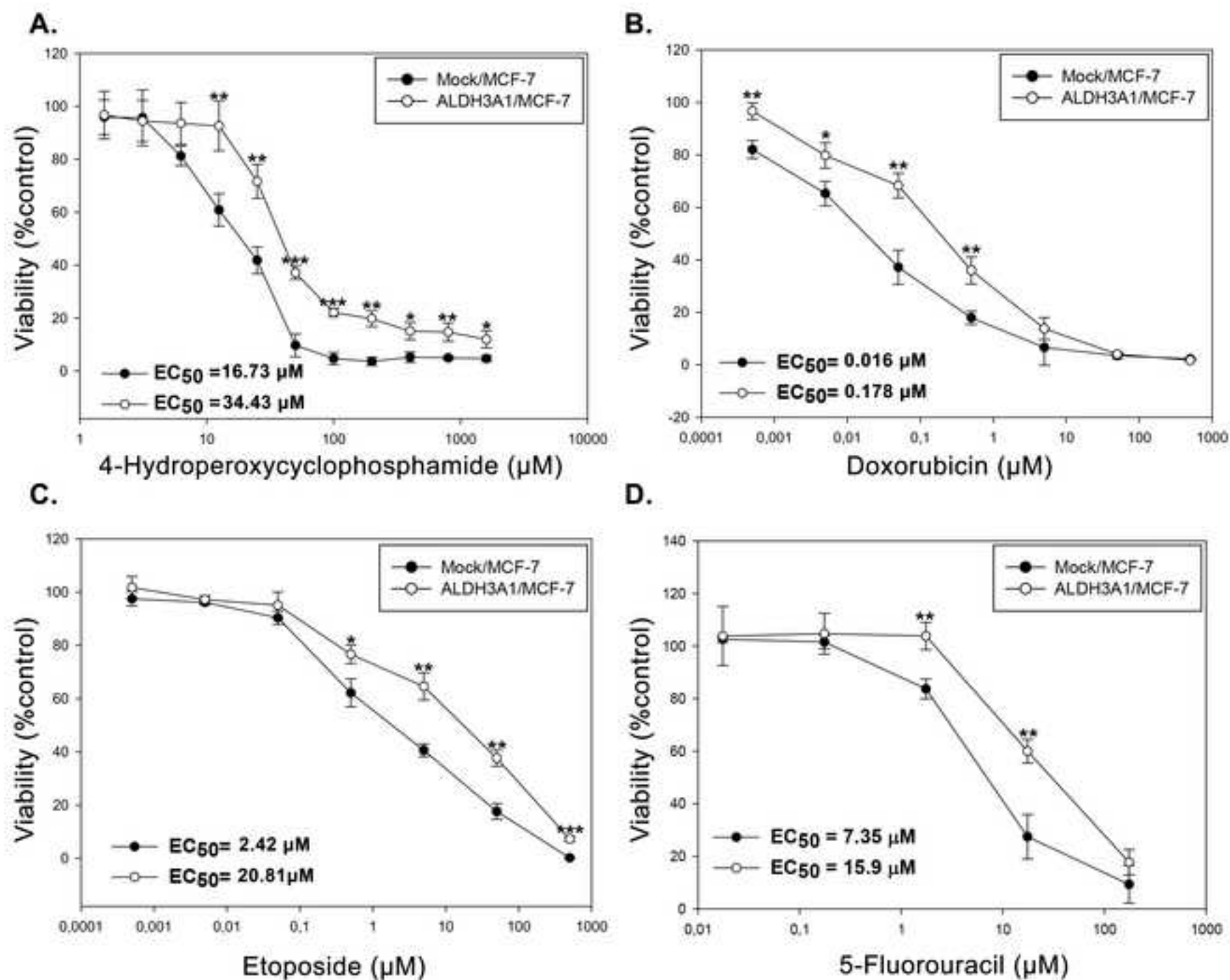
Figure(s)

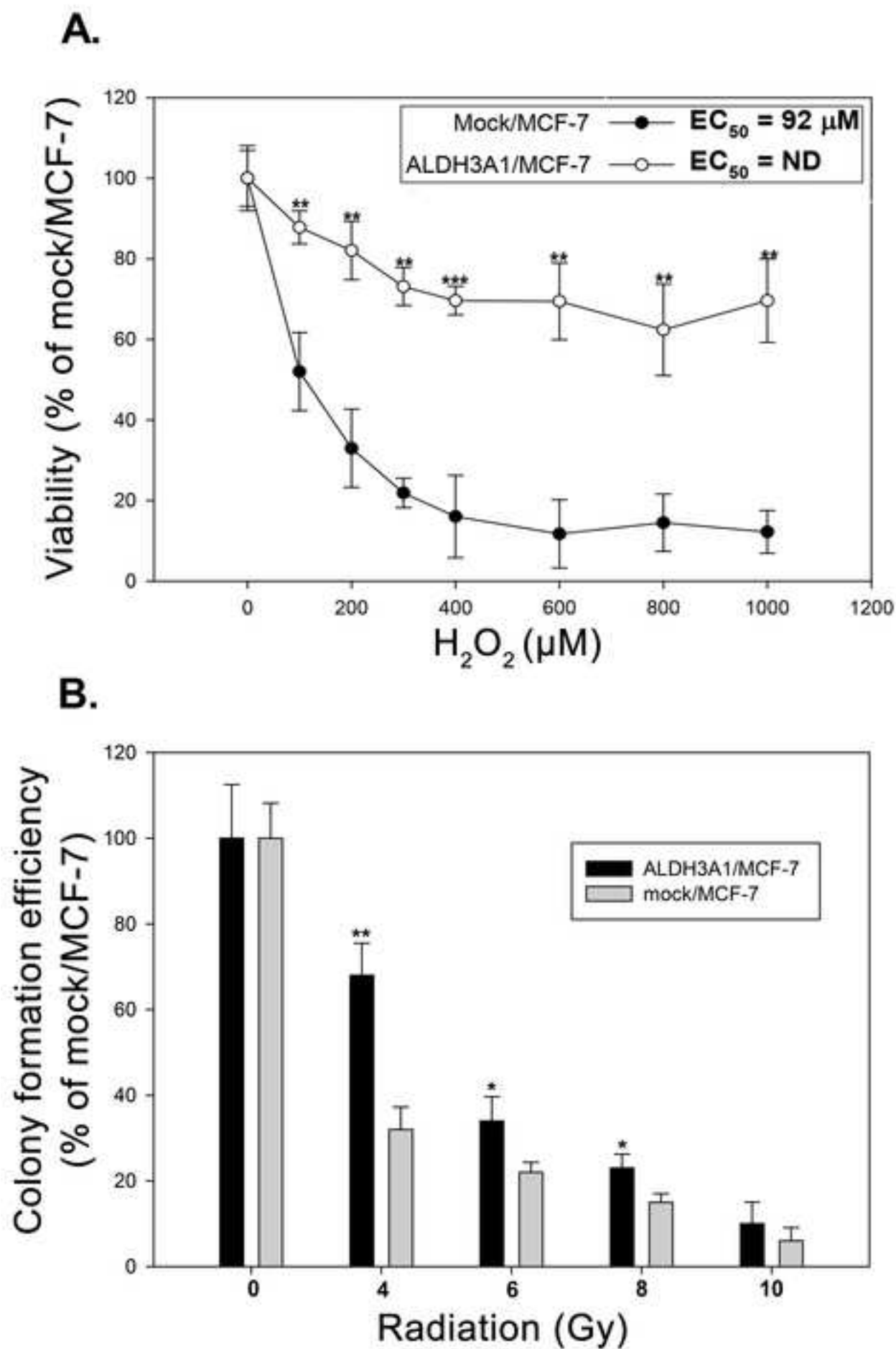
[Click here to download high resolution image](#)



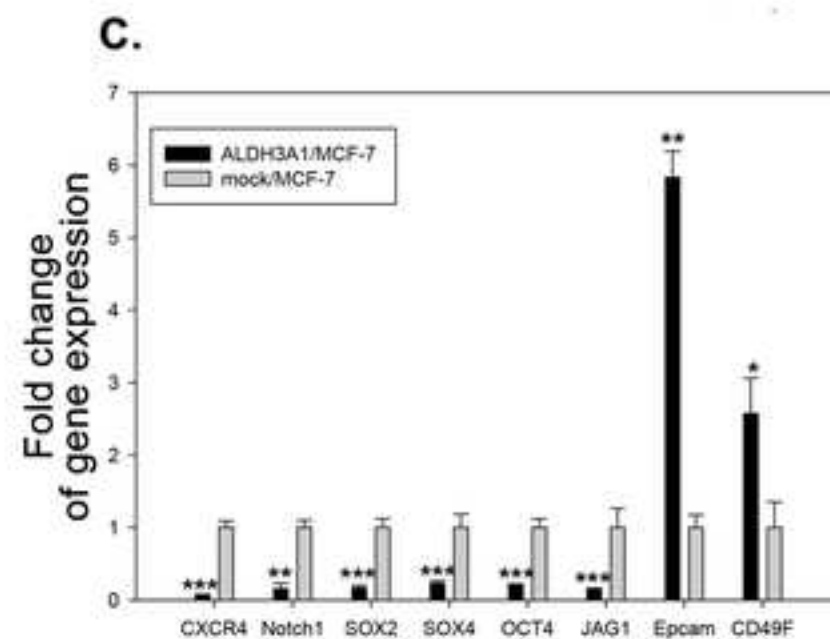
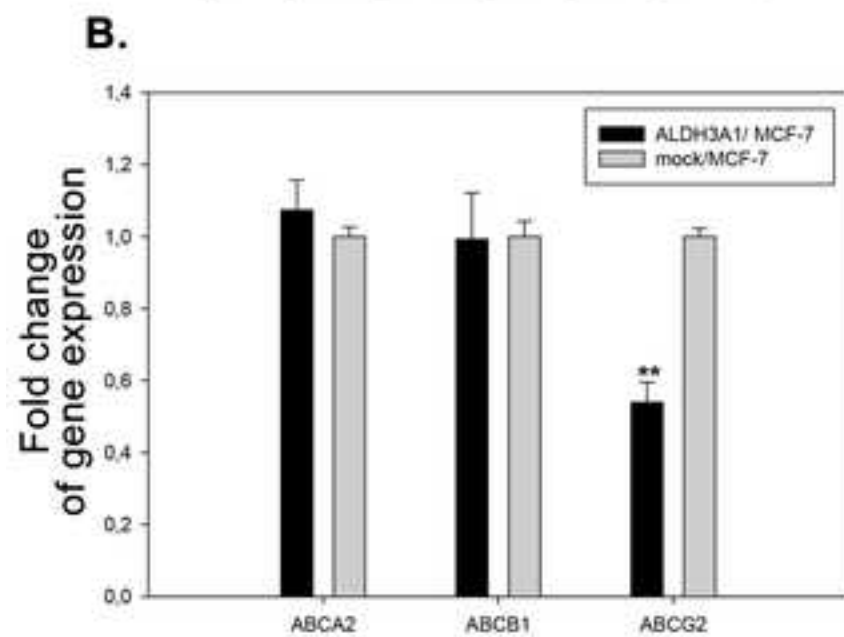
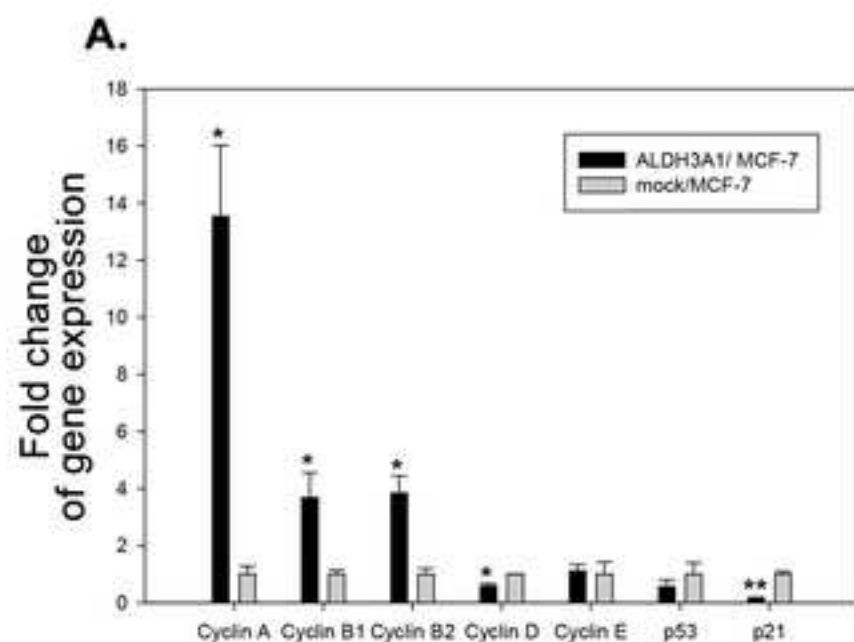
Figure(s)

[Click here to download high resolution image](#)

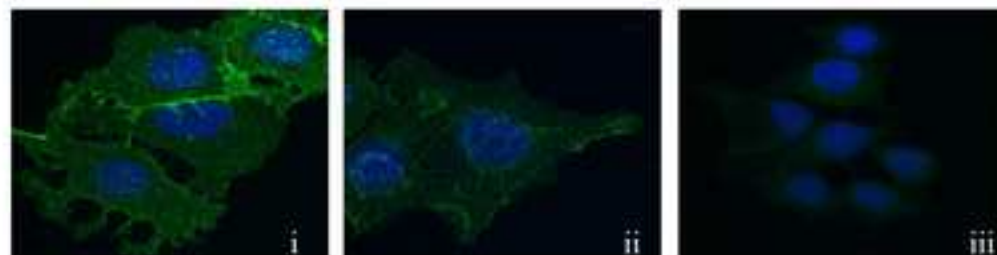




Figure(s)
[Click here to download high resolution image](#)



D.



E.

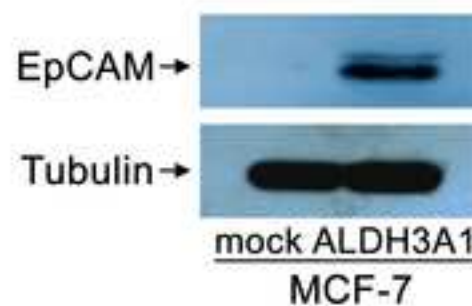


Table 1. Primers used for the real-time PCR comparative quantification

GENE	FORWARD PRIMER	REVERSE PRIMER
<i>β-actin</i>	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTC
<i>ALDH3A1</i>	CAGCGGCATGGGATCCTA	GCGGCGGTGAGAGAAAGTC
<i>Cyclin A</i>	ACGGGTTGCACCCCTTAAG	CCAAGGAGGAACGGTGACA
<i>Cyclin B1</i>	GGCCTCTACCTTTGCACTTCCT	GCTCGACATCAACCTCTCCAA
<i>Cyclin B2</i>	AAGCTTTTTCTGATGCCTTGCT	AGGGTTCTCCAATCTTCGTTAT
<i>Cyclin D</i>	AGACCTTCGTTGCCTCTTG TG	ATGGAGGGCGGATTGGAA
<i>Cyclin E</i>	GGCCTTGTATCATTCTCGTCAT	CGCACCCTGATACCCTGAA
<i>p53</i>	TCTGTCCCTTCCAGAAAACC	CAAGAAGCCCAGACGGAAAC
<i>p21</i>	GGCGGGCTGCATCCA	AGTGGTGTCTCGGTGACAAAGTC
<i>ABCA2</i>	AGATGGACAAGATGATCGAG	GCTTGACTTCAGGATGAGG
<i>ABCB1</i>	GAGGAAGACATGACCAGGTA	CTGTCGATTATAGCATGAA
<i>ABCG2</i>	ACCTGAAGGCATTTACTGAA	TCTTCCTTGCAGCTAAGAC
<i>CXCR4</i>	GGCCGACCTCCTCTTTGTC	TTGCCACGGCATCAACTG
<i>Notch1</i>	GCACCTCAGCCTGCACAGT	CTGTGTTGCTGGAGCATCTTCT

<i>SOX2</i>	TGCGAGCGCTGCACAT	TCATGAGCGTCTTGGTTTTCC
<i>SOX4</i>	CTGCGCCTCAAGCACATG	TTCTTCCTGGGCCGGTACT
<i>Oct4</i>	CGACCATCTGCCGCTTTG	GCCGCAGCTTACACATGTTCT
<i>JAG1</i>	TGAAGTAGAAGAGGACGACATGGA	CGGCTGCTTGGCAAACC
<i>EpCAM</i>	TTATGATCCTGACTGCGATGAGA	GGTGCCGTTGCACTGCTT
<i>CD49F</i>	GATCCCGGCCTGTGATTAATATT	CTGGCGGAGGTCAATTCTGT